Original Article

Dihydroorotate dehydrogenase promotes cell proliferation and suppresses cell death in esophageal squamous cell carcinoma and colorectal carcinoma

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Background: Ferroptosis is defined as an iron-dependent non-apoptotic form of programmed cell death. Dihydroorotate dehydrogenase (DHODH) is a newly discovered anti-ferroptosis molecule independent from the well-known GPX4 and AIFM2. However, the expression pattern and especially the functional roles of DHODH during cancer cell death are generally unknown.

Methods: The databases of Gene Expression Profiling Interactive Analysis (GEPIA), Kaplan-Meier Plotter, and Tumor Immune Estimation Resource (TIMER), and methods of colony formation, Cell Counting Kit-8 (CCK-8), adenosine triphosphate (ATP) detection, RNA-seq, quantitative reverse transcription polymerase chain reaction (qRT-PCR), and western blotting were used to analyze the expression level, prognostic role, and oncogenic roles of DHODH in cancers.

Results: DHODH overexpression was identified in many types of cancers including esophageal carcinoma (ESCA), colon adenocarcinoma (COAD), rectum adenocarcinoma (READ), and so on. Silence and inactivation of DHODH decreased the abilities of cell proliferation, colony formation, and cellular ATP levels both in esophageal squamous cell carcinoma (ESCC) and colorectal cancer (CRC) cells. Z-VAD-FMK (an apoptosis inhibitor) partially rescued blockade of DHODH-induced death of ESCC cells, and ferroptosis inhibitors (ferrostatin-1 and liproxstatin-1) together with the necroptosis inhibitor (necrostatin-1) partially rescued inhibition of DHODH-induced death of CRC cells, respectively. Pathways including rheumatoid arthritis, salmonella infection, cytokine-cytokine receptor interaction, pertussis, and nuclear factor-κB (NF-κB) were enriched in DHODH-silenced ESCC cells.

Conclusions: Overexpression of DHODH augments cell proliferation and suppresses cell death in ESCC and CRC, and DHODH might be developed as a potential anticancer target.

Keywords: Ferroptosis; apoptosis; dihydroorotate dehydrogenase (DHODH); esophageal squamous cell carcinoma (ESCC); colorectal carcinoma

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Introduction

As a newly-defined form of cell death, ferroptosis has the main characteristic of iron-dependent lipid peroxidation (1). Ferroptosis is regarded as a promising natural anti-tumor therapeutic method (2). AIFM2 and GPX4 have been identified as the 2 major ferroptosis defense systems (3-6). Dihydroorotate dehydrogenase (DHODH) is a newly discovered anti-ferroptosis molecule independent from GPX4 and AIFM2, the inactivation of which causes enhanced mitochondrial peroxidation and ferroptosis in GPX4-lowly-expressed cancer cells (7). However, the expression pattern, prognostic role, and especially the ferroptosis-related functional role of DHODH in pan-cancer are still largely unclear.

DHODH shows important roles during *de novo* pyrimidine biosynthesis, and its suppression induces apoptosis and differentiation of acute myeloid leukemia (AML) cells. Its inhibitor MEDS433 could synergically induce the metabolic lethality of AML cells with dipyridamole which is an inhibitor of the pyrimidine salvage pathway (8). In oral squamous cell carcinoma (OSCC), DHODH was found to be overexpressed and its elevated expression was significantly correlated with patients' unfavorable prognosis. Besides, inactivation of DHODH by either inhibitor or short hairpin RNA (shRNA) was shown to impede OSCC cell proliferation *in vitro* and tumor growth *in vivo* (9). In esophageal squamous cell carcinoma (ESCC), DHODH directly bound to the NH2 terminal of β-catenin, inhibited its protein degradation, and promoted its nuclear accumulation, then finally activated the β-catenin signaling pathway (10). DHODH together with PCK1 was found to promote the liver metastatic colonization and hypoxic growth of colorectal cancer (CRC) via enhancing nucleotide synthesis (11). Recent studies have reported that blocking SOD2 could enhance ferroptosis and augment radiosensitivity of nasopharyngeal carcinoma via regulating DHODH activity, and that suppression of DHODH synergistically improved therapeutic effect with cisplatin in cervical cancer via inducting ferroptosis (12,13). However, the roles of DHODH in the ferroptosis of ESCC and CRC cells are still unclear.

In the present study, we analyzed the expression level and prognostic value of DHODH in cancers, and importantly explored the roles of DHODH in the proliferation and cell death of ESCC and CRC cells. We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-136/rc).

Highlight box

Key findings

- Dihydroorotate dehydrogenase (DHODH) was overexpressed in esophageal carcinoma (ESCA), colon adenocarcinoma (COAD), and rectum adenocarcinoma (READ). Silence and inactivation of DHODH inhibited the proliferation and induced cell death of esophageal squamous cell carcinoma (ESCC) and colorectal cancer (CRC) cells.

What is known and what is new?

- DHODH is newly identified as an anti-ferroptosis molecule independent from the well-known GPX4 and AIFM2.
- Silence and inactivation of DHODH inhibited the proliferation and induced cell death of ESCC and CRC cells.

What is the implication, and what should change now?

- Targeting DHODH-induced ferroptosis will probably be used to treat DHODH-overexpressed cancers in future.

Methods

Patients and tissues

A total of 20 surgical resected ESCC tissues were collected at First People's Hospital of Yunnan Province and Affiliated Hospital of Kunming University of Science and Technology (Kunming, China). All participants had not received treatment before surgery and provided informed consent. The Medical Ethics Committee of Kunming University of Science and Technology approved this study (No. KMUST-MEC-123). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Cell lines, transfection, and reagents

KYSE450 and KYSE510 ESCC and SW620 CRC cell lines were cultured in Roswell Park Memorial Institute (RPMI; Buffalo, NY, USA) 1640 cell culture medium [containing

10% fetal bovine serum (FBS), 100 mg/mL streptomycin, and 100 U/mL penicillin] under the conditions of 37 ℃ and 5% CO_2. Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) was applied for cell transfection.

DHODH and negative control small interfering RNAs (siRNAs) were produced by GenePharma (Shanghai, China). Leflunomide (LEF), Z-VAD-FMK, necrostatin-1, liproxstatin-1, and ferrostatin-1 were provided by Selleck (Houston, TX, USA).

Total RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Previous methods were applied to extract total RNA and perform qRT-PCR assay (14). Details of the primers used are provided in Table S1.

Western blot assay

A previously described method was applied for western blot analysis (14). The antibodies were as follows: DHODH antibody (14877-1-AP; Proteintech, Wuhan, China), GPX4 antibody (ab125066; Abcam, Cambridge, MA, USA), SLC7A11 antibody (ab175186; Abcam), and GAPDH antibody (60004-1-IG; Proteintech).

Cell viability, proliferation, colony formation, and cellular adenosine triphosphate (ATP) assays

Previous methods were applied to detect cell viability and abilities of cell proliferation and colony formation (14). Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Tokyo, Japan) was harnessed to measure cell viability and proliferation ability. In colony formation, clones were stained by crystal violet (Sigma Aldrich, St. Louis, MO, USA) and further visualized and quantitated. Cellular ATP levels were detected using a Beyotime enhanced ATP assay kit (Beyotime, Shanghai, China).

Messenger RNA (mRNA) sequencing

RNA extraction and mRNA sequencing were performed by BGI Genomics Co., Ltd. (Shenzhen, China). Total RNA was prepared using TRIzol™ reagent (Thermo Fisher). An Agilent 2100 Bioanalyzer system (Santa Clara, CA, USA) was harnessed to measure RNA quantity and quality using an RNA Nano 6000 Kit. The mRNAs from samples were sequenced by DNBSEQ platform (MGI Tech., Shenzhen, China).

Differentially expressed genes (DEGs) were identified through comparing normalized reads count between DHODH-silenced and control cells with absolute $\log_2$ [fold change (FC)]>1.0 and P<0.05.

Databases analyses

The Kaplan-Meier Plotter (15), Tumor Immune Estimation Resource (TIMER) (16-18), Gene Expression Profiling Interactive Analysis (GEPIA) (19) databases were applied to analyze expression pattern, prognostic role, and correlations between indicated genes in pan-cancer.

Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses

KEGG pathway enrichment and GO analyses were carried out on the Database for Annotation, Visualization and Integrated Discovery (DAVID) platform (20,21).

Statistical analyses

Statistical analysis was carried out through GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Data were shown as mean ± standard deviation (SD) and analyzed by analysis of variance (ANOVA) and Student's *t*-test. Kaplan-Meier method was used to estimate overall survival (OS) time. Statistical significance was considered when P<0.05.

Results

Pan-cancer analysis of DHODH expression pattern and its prognostic role

Analysis of the TIMER database revealed that DHODH was overexpressed in many types of cancers such as esophageal carcinoma (ESCA), colon adenocarcinoma (COAD), and rectum adenocarcinoma (READ). Using the GEPIA database, DHODH expression was found to be lower in liver hepatocellular carcinoma (LIHC) and higher in diffuse large B-cell lymphoma (DLBC), glioblastoma (GBM), thymoma (THYM), and lower grade glioma (LGG) comparing with corresponding normal tissues (Figure S1A,S1B).

Pan-cancer prognosis analysis of DHODH was achieved via analyzing the Kaplan-Meier Plotter, GEPIA, and TIMER databases. High expression of DHODH was correlated with improved OS and relapse-free survival

(RFS) of kidney renal papillary cell carcinoma (KIRP) and lung adenocarcinoma (LUAD) patients, and associated with shortened OS as well as RFS of sarcoma (SARC) patients (Figure S2A-S2F). In the TIMER database, DHODH high expression indicated poor prognosis in SARC but had no prognostic value in KIRP (Figure S2G,S2H). In the Kaplan-Meier Plotter database, DHODH high expression was positively linked with good OS in liver cancer, READ, THYM, and uterine corpus endometrial carcinoma (UCEC), and poor RFS of cervical squamous cell carcinoma (CSCC) and stomach adenocarcinoma (STAD) patients, and improved RFS in thyroid cancer (THCA) (Figure S2I-S2O). In the GEPIA database, correlations between DHODH high expression and good OS in KIRC or poor disease-free survival (DFS) in pancreatic adenocarcinoma (PAAD) or improved DFS in LIHC were identified (Figure S3A-S3D).

Interestingly, after analyzing the TIMER database, data showed that DHODH mRNA expression was higher in *TP53*-mutated bladder urothelial carcinoma (BLCA), ESCA, human papillomavirus (HPV)-negative head and neck squamous cell carcinoma (HNSC), luminal-A breast invasive carcinoma (BRCA), and lower in *TP53*-mutated uterine carcinosarcoma (UCS) and LIHC (Figure S4A-S4F), and also higher in *PTEN*-mutated KIRP, prostate adenocarcinoma (PRAD), UCEC, and BLCA (Figure S5A-S5D).

Considering that DHODH was overexpressed in GBM and under-expressed in LIHC both in GEPIA and TIMER databases, GO and KEGG analyses were further performed based on DEGs both in GBM [n=500, Pearson correlation coefficient (PCC): 0.79–0.57] and LIHC (n=100, PCC: 0.73–0.33) by DAVID. In GBM, biological process (BP) enrichment analysis indicated that DEGs were correlated with transcription, mRNA splicing, and so on (Figure S6A and Table S2); cellular component (CC) enrichment analysis indicated that DEGs were correlated with nucleoplasm, nucleus, and so on (Figure S6B and Table S3); molecular function (MF) enrichment analysis indicated that DEGs were correlated with nucleic acid binding, poly (A) RNA binding, and so on (Figure S6C and Table S4); KEGG enrichment analysis indicated that DEGs were correlated with spliceosome, mRNA surveillance pathway, and so on (Figure S6D and Table S5). In LIHC, BP were enriched in acute-phase response and so on (Figure S6E and Table S6); CC were enriched in blood microparticle and so on (Figure S6F and Table S7); MF were enriched in serine-type endopeptidase activity and so on (Figure S6G and Table S8); KEGG analysis indicated that DEGs were enriched in complement and coagulation cascades and so on (Figure S6H and Table S9).

Then, we evaluated the associations between DHODH and ferroptosis-associated genes including *GPX4*, *SLC7A11*, and *AIFM2* using the GEPIA database, and positive correlation ($P<0.05$ and $R\geq0.2$) suggested that DHODH might participate in ferroptosis by cross-linking with *GPX4*/*SLC7A11*/*AIFM2*. Positive correlations between DHODH and *GPX4* were detected in ESCA (P=0.002, R=0.230) together with kidney chromophobe (KICH; P=0.003, R=0.360), and between DHODH and *SLC7A11* in many types of cancers such as GBM and lung squamous cell carcinoma (LUSC), Table S10), and between DHODH and *AIFM2* in CESC, ESCA, and so on (Table S10).

DHODH was overexpressed in ESCC and CRC

Figure 1 shows that DHODH was overexpressed in ESCA, COAD, and READ in mRNA level. Overexpression of DHODH in ESCC and CRC was further validated via analyzing ESCC datasets (GSE20347, GSE53622, and GSE53624; *Figure 2A-2C*) and CRC datasets (GSE24514 and GSE4107; *Figure 2D*,*2E*). Importantly, DHODH high expression was markedly associated with unfavorable outcome in CRC (*Figure 2F*). Expression of DHODH was positively correlated with SLC7A11 mRNA level both in GSE53622 and GSE53624 datasets, and correlation between expressions of DHODH and GPX4 were only identified in GSE53624 dataset, and there was no correlation between expressions of SLC7A11 and GPX4 in 2 datasets (*Figure 2G-2L*). The qRT-PCR assay verified higher expression of DHODH in ESCC samples comparing to normal tissues (*Figure 2M*, n=20). Using western blot, protein expressions of DHODH, SLC7A11, and GPX4 in ESCC tissues were detected, moreover DHODH, SLC7A11, and GPX4 were overexpressed in 3, 4, and 2 ESCC samples respectively (ratio ≥1.5; *Figure 2N*).

Silence or inactivation of DHODH inhibited the proliferation and induced the cell death in ESCC and CRC cells

Knockdown of DHODH remarkably suppressed ESCC (KYSE510 and KYSE450) and CRC (SW620) cells' proliferation (*Figure 3A-3D*). Colony formation abilities of DHODH-silenced KYSE510 and KYSE450 cells were descended (*Figure 3E*). Interestingly, knockdown of DHODH decreased the cellular ATP levels in KYSE510, KYSE450, and SW620 cells (*Figure 3F-3H*).

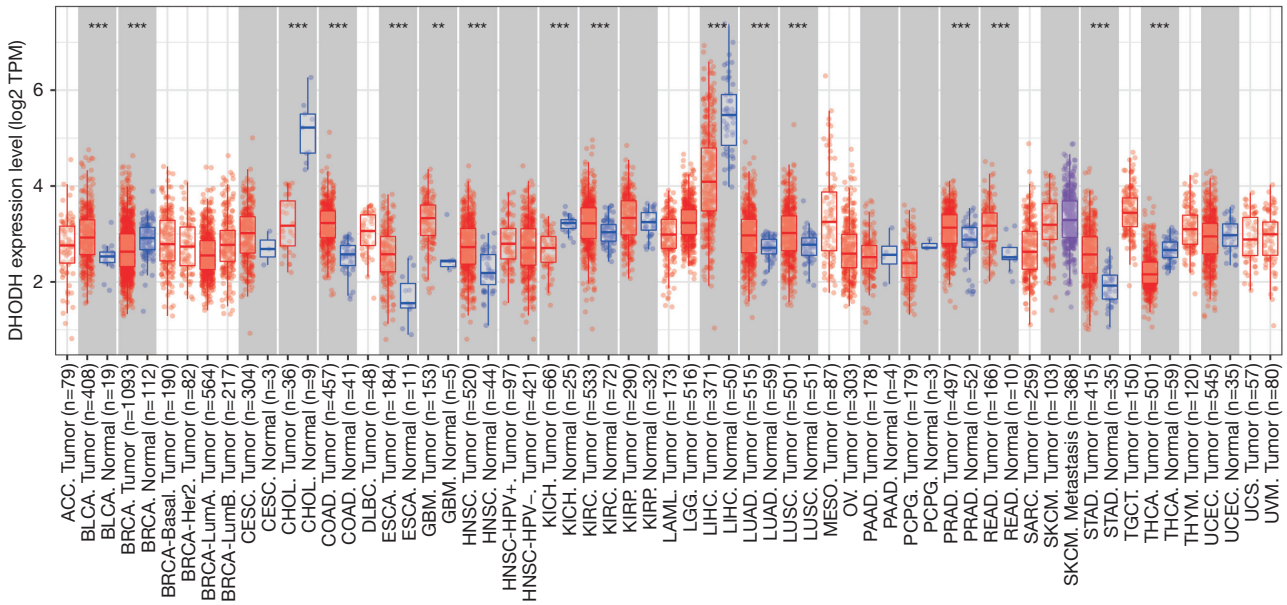

Figure 1 Pan-cancer analysis of DHODH expression pattern. Expression pattern was analyzed by using TIMER database. **, P<0.01; ***, P<0.001. DHODH, dihydroorotate dehydrogenase; TPM, transcripts per million; TIMER, Tumor Immune Estimation Resource.

LEF, a clinically approved DHODH inhibitor, was harnessed to evaluate the influences of blocking DHODH on the phenotypes of cancer cells. The IC50 of LEF in KYSE510, KYSE450, and SW620 cells was 108.2 μM, 124.8 μM, and 173.9 μM, respectively (*Figure 4A-4C*). LEF significantly reduced viabilities of KYSE510, KYSE450, and SW620 cells (*Figure 4D*). Colony formation assay showed that LEF treatment markedly reduced the colony formation abilities of both ESCC cells and CRC cells (*Figure 4E,4F*). LEF also significantly reduced the cellular ATP levels in KYSE510, KYSE450, and SW620 cells (*Figure 4G*). Importantly, the inhibitory effects on ESCC cell viabilities of LEF could be partially recovered by the inhibitor of apoptosis Z-VAD-FMK, moreover the inhibition on cell viability of CRC cells caused by LEF could be partially impeded by ferroptosis inhibitors (Lip-1, Liproxstatin-1; Fer-1, Ferrostatin-1) and necroptosis inhibitor (Nec-1, Necrostatin-1; *Figure 4H–4J*). These results suggested that DHODH promoted the proliferation and suppressed the cell death both in ESCC and CRC.

Identification of downstream target genes and pathways of DHODH in ESCC

DEGs after DHODH knockdown were screened by using RNA-seq method. After DHODH knockdown, 27 genes were downregulated and 14 genes were upregulated both in KYSE510 and KYSE450 cells (*Figure 5A-5C* and *Table 1*). The top 5 downregulated genes were *U2AF1*, *SULT1A4*, *EIF3CL*, *DHODH*, and *TXNDC12*, and the top 5 upregulated genes were *H4C12*, *SDR16C5*, *GDF15*, *TMED7-TICAM2*, and *GPR75*. The CC of extracellular space was enriched both in KYSE510 and KYSE450 cells (*Figure 5D,5E*). There was no common BP both in KYSE510 and KYSE450 (*Figure 5F,5G*). The MF of growth factor activity was enriched both in KYSE510 and KYSE450 cells (*Figure 6A,6B*). The enriched pathways were rheumatoid arthritis, salmonella infection, cytokine-cytokine receptor interaction, pertussis, and nuclear factor-κB (NF-κB) (*Figure 6C,6D*). In qRT-PCR validation experiment, silence of DHODH downregulated TXNDC, USE1, and U2AF1 and upregulated HMOX1 and PTPRB both in KYSE510 and KYSE450 cells (*Figure 6E,6F*). Besides that, LEF treatment also significantly decreased the mRNA levels of DHODH, TXNDC12, USE1, and U2AF1 both in KYSE510 and KYSE 450 cells (*Figure 6G,6H*).

Discussion

DHODH plays an important role in *de novo* pyrimidine synthesis and is a flavin-dependent mitochondrial enzyme (22). The synthesis of pyrimidine nucleotides

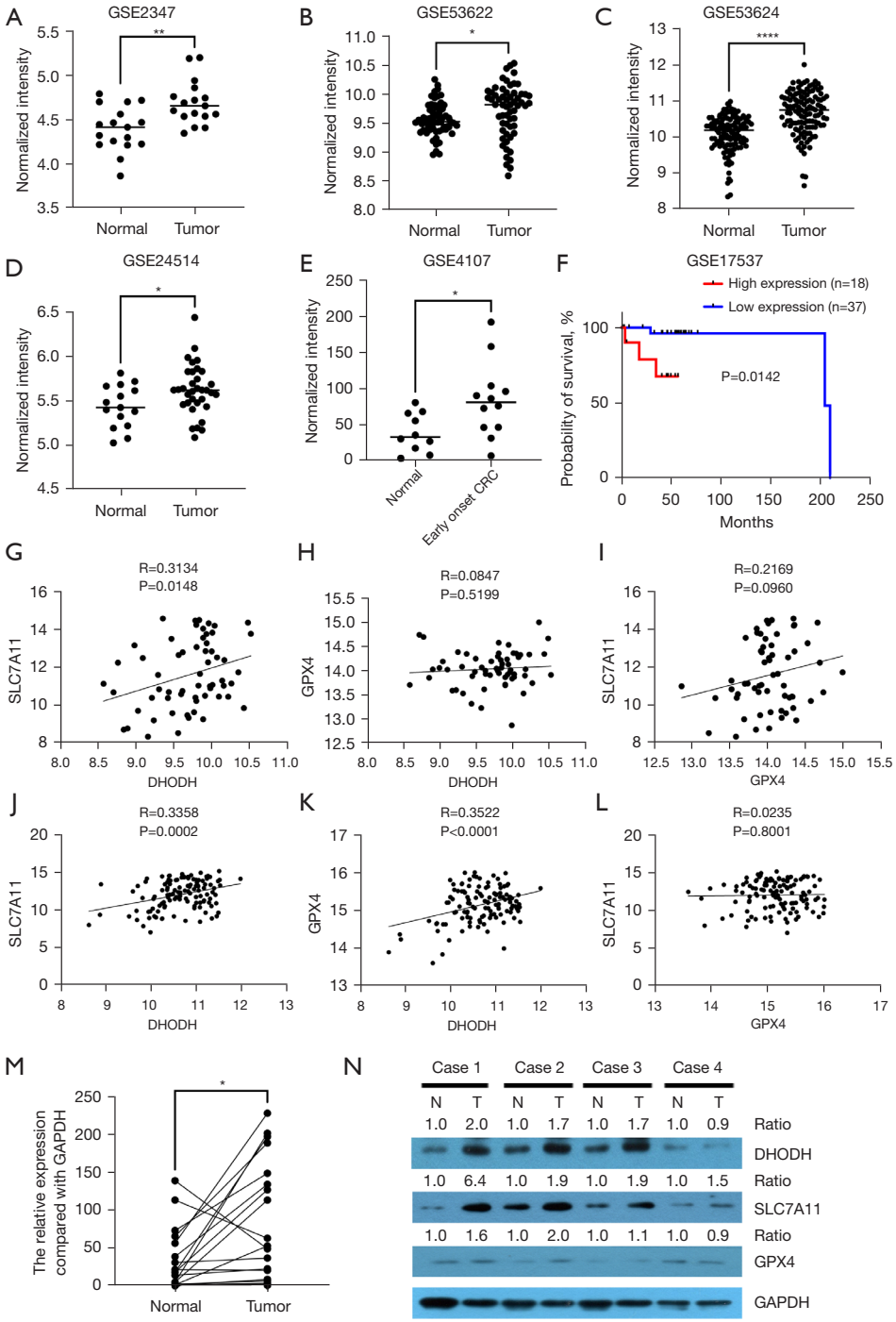

Figure 2 Overexpression of DHODH in ESCC and CRC. (A-E) Transcript levels of DHODH in ESCC datasets of GSE20347, GSE53622, and GSE53624, and in CRC datasets of GSE24514 and GSE4107. (F) Prognostic correlation of DHODH in CRC was analyzed using GSE17537. (G-I) Correlations were analyzed using the GSE53622 dataset. (J-L) Correlations were analyzed using the GSE53624 dataset. (M) The mRNA expression of DHODH was analyzed in ESCC samples using qRT-PCR assay (n=20; mean ratio of tumor/normal=8.03). (N) The protein expression levels of DHODH and ferroptosis associated genes SLC7A11 and GPX4 were analyzed in ESCC tissues (n=4) using Western blot assay. Ratio = [intensity of DHODH/intensity of GAPDH (tumor or normal)]/[intensity of DHODH/intensity of GAPDH (normal)]. *, P<0.05; **, P<0.01; ****, P<0.0001. DHODH, dihydroorotate dehydrogenase; ESCC, esophageal squamous cell carcinoma; CRC, colorectal cancer; mRNA, messenger RNA; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

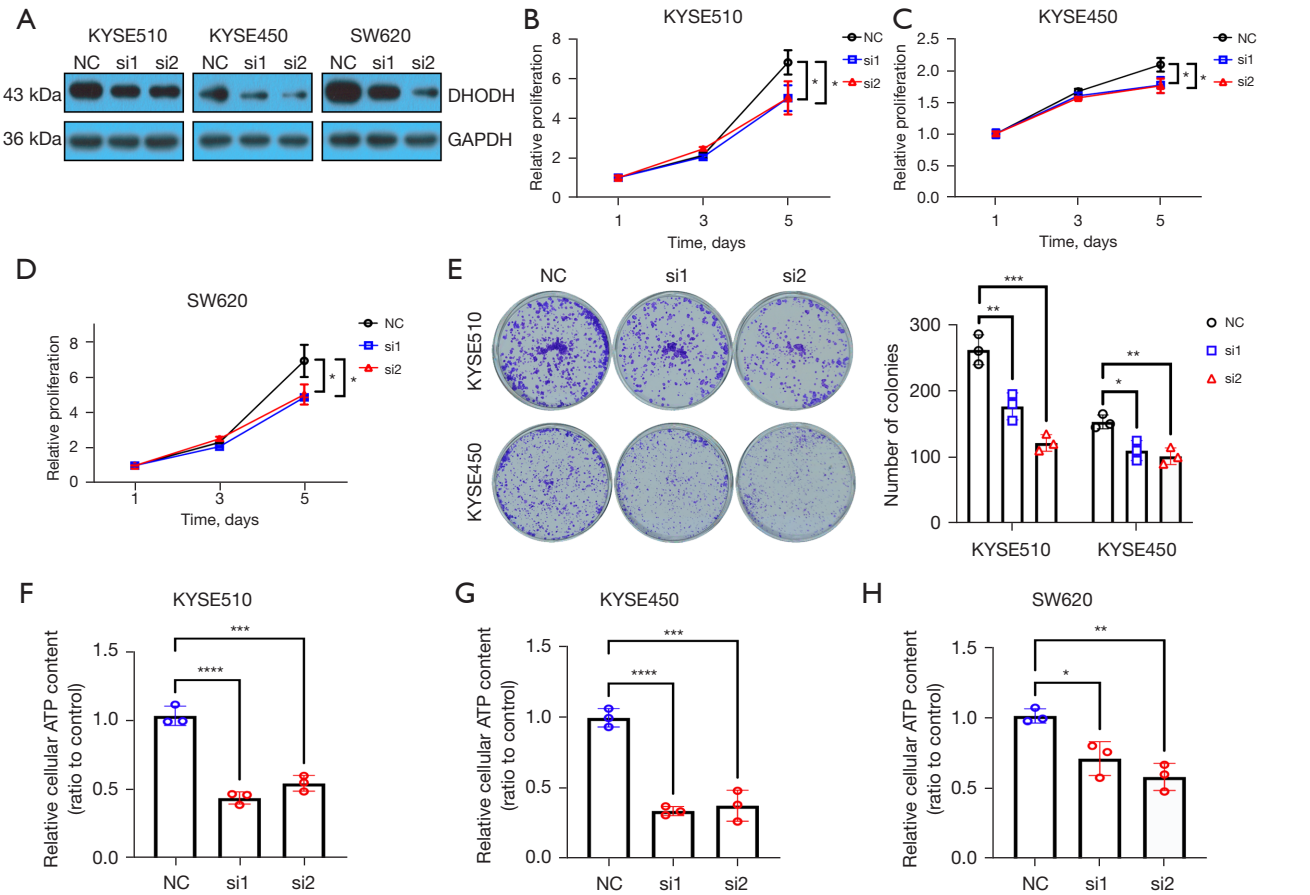

Figure 3 Silence of DHODH suppressed colony formation, proliferation, and reduced cellular ATP levels in ESCC and CRC. (A) The efficiency of DHODH knockdown was detected using western blot assay. (B-E) CCK-8 together with colony formation methods were applied to detect the influences of DHODH knockdown on the ESCC and CRC cells' proliferation and colony formation abilities. (F-H) Influences of DHODH knockdown on cellular ATP levels in ESCC and CRC cells were measured. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. NC, negative control; DHODH, dihydroorotate dehydrogenase; ATP, adenosine triphosphate; ESCC, esophageal squamous cell carcinoma; CRC, colorectal cancer; CCK-8, Cell Counting Kit-8.

consists of 2 pathways including the *de novo* pathway and salvage pathway. In rapidly proliferative cells, pyrimidine nucleotides are mainly synthesized by the *de novo* pathway, and in fully differentiated cells, pyrimidines nucleotides are mainly obtained by the salvage pathway (23,24). DHODH is located in mitochondria inner membrane because the N-terminal of human DHODH protein contains the mitochondrial-targeting pre-sequences (25). However, recent research has reported that DHODH functioned as a new anti-ferroptosis gene independent from *GPX4* and *AIFM2* via affecting the mitochondrial lipid peroxidation (7). Delivering multi-siRNAs targeting GPX4 and DHODH using exosomes could augment sorafenib-induced ferroptosis of hepatocellular carcinoma cells (26). Manganese treatment could inhibit DHODH activity via regulating type-I interferons (IFNs) and eventually lead to ferroptosis of cancer cells (27). However, its roles and mechanisms in the ferroptosis of many other cancers, especially ESCC and CRC, are still largely unclear, and whether its anti-ferroptosis function is associated with its roles in *de novo* pyrimidine synthesis or its location in mitochondria inner membrane is still unknown.

Ferroptosis has been shown to participate in the development and therapeutic responses of many kinds of tumors, and damage of ferroptosis could induce inflammation-linked immunosuppression in the microenvironment of cancer and promote cancer growth (1). In the GEPIA and TIMER databases, DHODH was

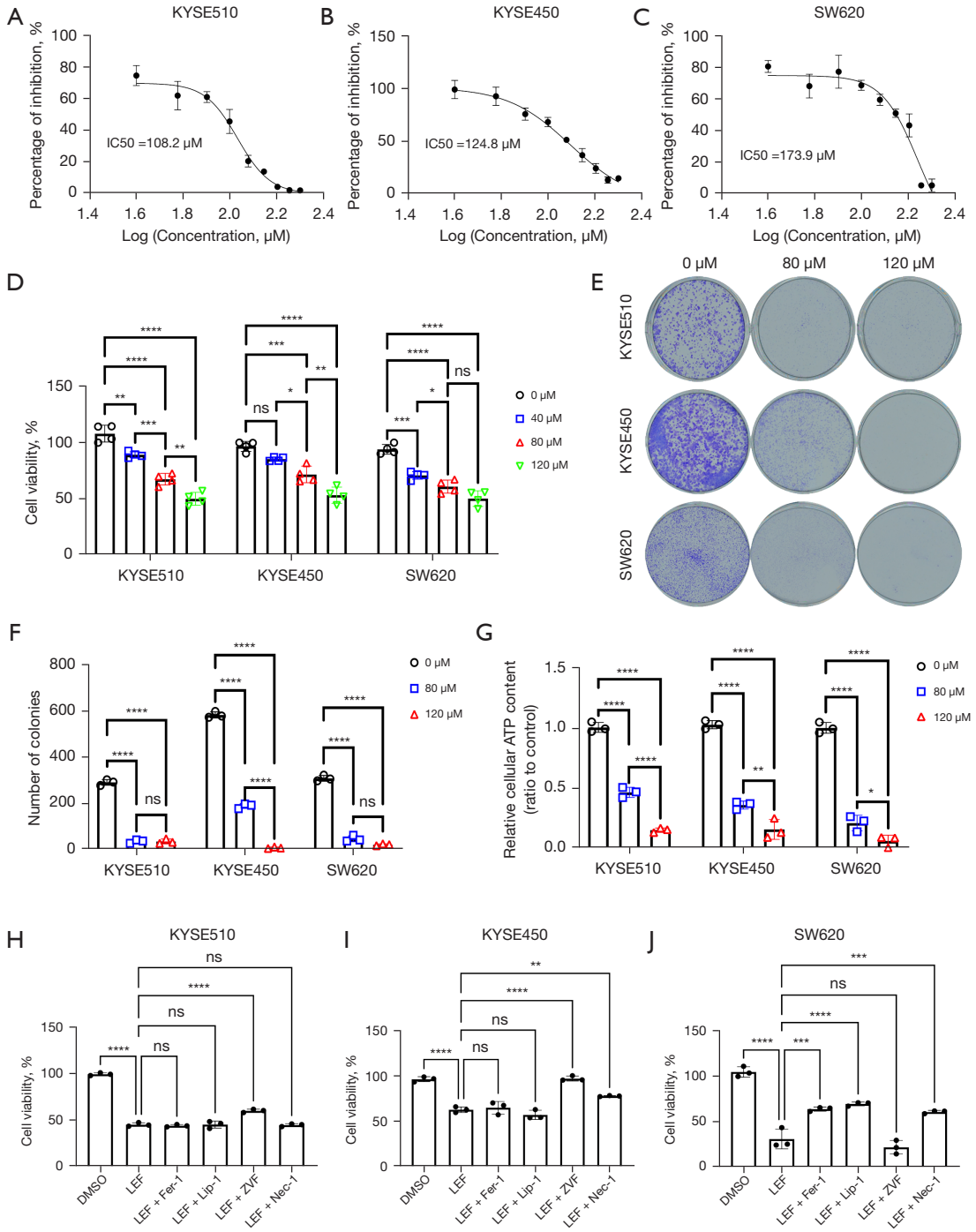

Figure 4 DHODH inhibitor reduced viability and colony formation ability of tumor cells and induced cell death in ESCC and CRC. (A-C) The IC50 of DHODH inhibitor LEF in KYSE510, KYSE450, and SW620 cells was measured. (D-F) CCK-8 as well as colony formation methods were employed to detect the influences of DHODH inhibitor on ESCC and CRC cells' proliferation. In the colony formation assay, clones were stained by crystal violet. (G) Cellular ATP levels in ESCC and CRC cells were measured. (H-J) The effects of necroptosis inhibitor (Necrostatin-1, Nec-1, 10 μM), apoptosis inhibitor (Z-VAD-FMK, ZVF, 20 μM) and ferroptosis inhibitors (Liproxstatin-1, Lip-1, 2 μM; Ferrostatin-1, Fer-1, 2 μM) on LEF-induced cell death were detected. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. DHODH, dihydroorotate dehydrogenase; ESCC, esophageal squamous cell carcinoma; CRC, colorectal cancer; IC50, half maximal inhibitory concentration; LEF, leflunomide; ATP, adenosine triphosphate; ns, not significant.

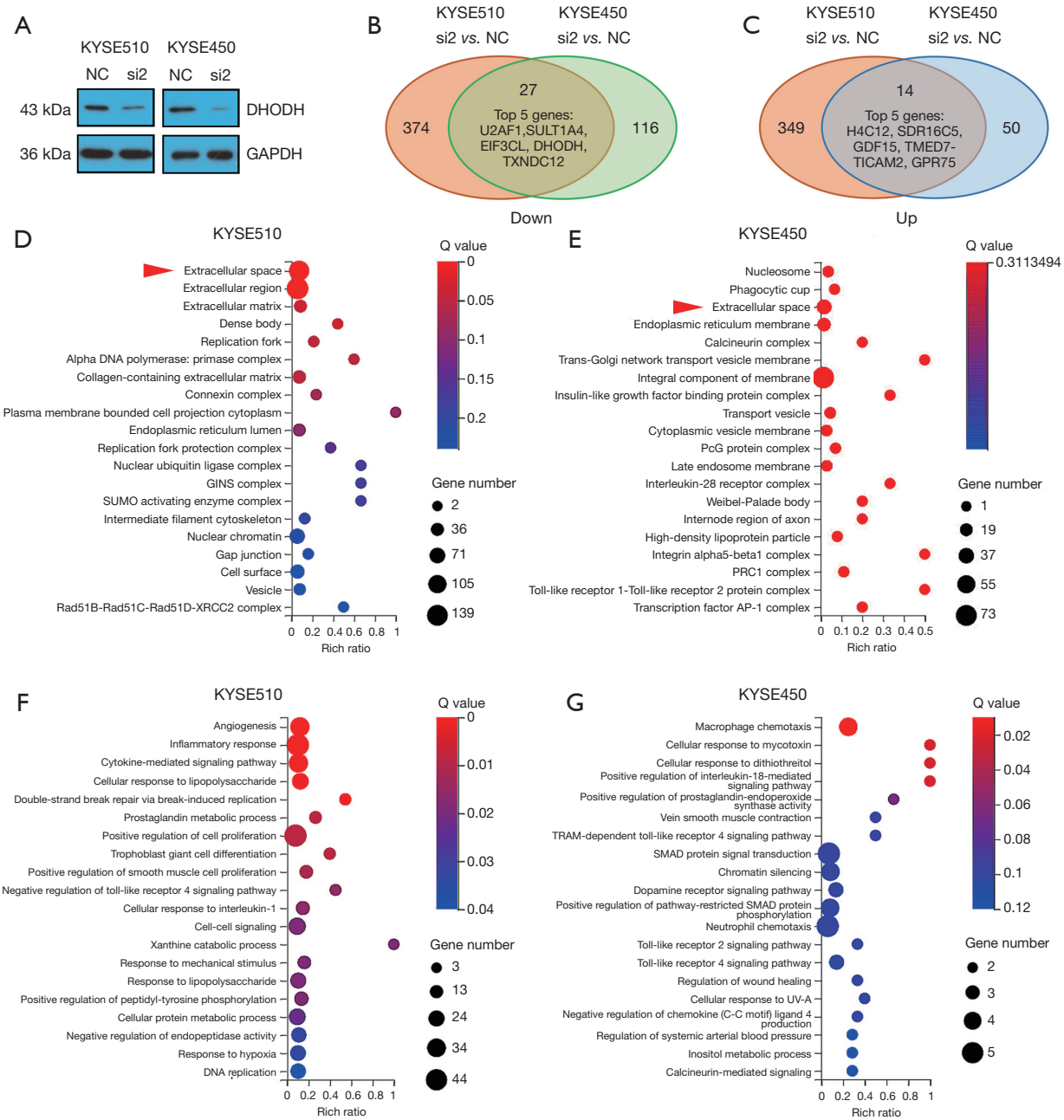

Figure 5 Identification of downstream target genes of DHODH in ESCC. (A) Efficiency of DHODH knockdown in KYSE510 and KYSE450 cells was confirmed using western blot assay. (B,C) Venn plot showed the overlapped downregulated and upregulated genes after DHODH knockdown in KYSE510 and KYSE450 cells. (D,E) CC analysis based on DEGs after DHODH knockdown in KYSE510 and KYSE450 cells. (F,G) BP analysis based on DEGs after DHODH knockdown in KYSE510 and KYSE450 cells. Arrowhead, enrichment in both KYSE510 and KYSE450 cells. NC, negative control; DHODH, dihydroorotate dehydrogenase; ESCC, esophageal squamous cell carcinoma; BP, biological process; DEG, differentially expressed gene; CC, cellular component.

Table 1 DEGs at the downstream of DHODH were identified the by using RNA-seq method

No.	Gene ID	Gene symbol	Regulation	KYSE450		KYSE510	
				log_2 (si2/NC)	FDR (si2/NC)	log_2 (si2/NC)	FDR (si2/NC)
1	8362	*H4C12*	Up	10.013	1.60E−04	9.392	6.32E−04
2	195814	*SDR16C5*	Up	2.921	4.27E−04	2.772	3.36E−28
3	9518	*GDF15*	Up	1.881	1.67E−87	1.843	0
4	100302736	*TMED7-TICAM2*	Up	1.846	1.50E−28	1.197	7.29E−21
5	10936	*GPR75*	Up	1.828	2.88E−05	1.143	2.60E−05
6	102800317	*TPTEP2-CSNK1E*	Up	1.732	2.71E−04	1.874	1.44E−06
7	55586	*MIOX*	Up	1.598	3.48E−04	3.949	4.50E−12
8	3162	*HMOX1*	Up	1.448	2.30E−08	2.949	0
9	5328	*PLAU*	Up	1.291	1.17E−124	1.332	5.49E−60
10	1839	*HBEGF*	Up	1.190	2.27E−17	2.852	0
11	5787	*PTPRB*	Up	1.142	2.49E−06	1.788	6.04E−05
12	79705	*LRRK1*	Up	1.099	0	1.136	2.39E−178
13	1073	*CFL2*	Up	1.078	3.77E−16	1.054	1.86E−40
14	118788	*PIK3AP1*	Up	1.011	3.26E−15	2.954	1.66E−07
15	7307	*U2AF1*	Down	−4.214	8.80E−109	−4.363	1.37E−218
16	445329	*SULT1A4*	Down	−2.324	2.43E−72	−10.329	9.97E−65
17	728689	*EIF3CL*	Down	−1.735	5.30E−252	−1.411	2.45E−35
18	1723	*DHODH*	Down	−1.634	1.98E−29	−2.548	6.52E−35
19	51060	*TXNDC12*	Down	−1.414	2.33E−80	−1.205	1.29E−84
20	65989	*DLK2*	Down	−1.374	1.05E−18	−1.306	2.37E−27
21	388963	*C2orf81*	Down	−1.336	3.85E−12	−1.724	7.76E−04
22	201266	*SLC39A11*	Down	−1.333	5.55E−20	−1.569	1.38E−12
23	1397	*CRIP2*	Down	−1.293	5.71E−08	−1.167	3.75E−11
24	5275	*SERPINB13*	Down	−1.241	1.03E−17	−1.201	6.52E−39
25	55646	*LYAR*	Down	−1.209	1.90E−41	−1.698	1.01E−85
26	1207	*CLNS1A*	Down	−1.197	3.84E−217	−1.934	4.40E−242
27	112495	*GTF3C6*	Down	−1.184	9.64E−11	−1.518	1.75E−44
28	114971	*PTPMT1*	Down	−1.160	4.78E−38	−1.419	9.39E−83
29	51529	*ANAPC11*	Down	−1.141	1.99E−25	−1.044	2.33E−21
30	55052	*MRPL20*	Down	−1.135	3.19E−22	−1.130	1.52E−42
31	10994	*ILVBL*	Down	−1.133	3.02E−35	−1.335	1.11E−37
32	79077	*DCTPP1*	Down	−1.111	2.83E−46	−1.562	2.88E−72
33	4059	*BCAM*	Down	−1.094	1.21E−26	−1.263	3.94E−34
34	2700	*GJA3*	Down	−1.063	1.43E−09	−1.190	8.21E−04

Table 1 (*continued*)

Table 1 (*continued*)

No.	Gene ID	Gene symbol	Regulation	KYSE450		KYSE510	
				log_2 (si2/NC)	FDR (si2/NC)	log_2 (si2/NC)	FDR (si2/NC)
35	285148	*IAH1*	Down	−1.062	2.93E−29	−2.027	2.97E−13
36	55850	*USE1*	Down	−1.037	1.34E−06	−1.035	3.94E−12
37	60558	*GUF1*	Down	−1.023	2.62E−37	−1.493	3.65E−59
38	54892	*NCAPG2*	Down	−1.020	1.16E−21	−1.624	7.03E−98
39	151188	*ARL6IP6*	Down	−1.008	5.69E−18	−1.256	7.80E−17
40	84992	*PIGY*	Down	−1.006	2.84E−04	−1.681	4.68E−20
41	5533	*PPP3CC*	Down	−1.005	2.37E−06	−1.690	4.75E−18

DEGs, differentially expressed genes; DHODH, dihydroorotate dehydrogenase; NC, negative control; FDR, false discovery rate.

overexpressed in GBM and under-expressed in LIHC. In high grade gliomas and GBM cell lines, DHODH was elevated, and in particular, inhibition of DHODH suppresses the proliferation of GBM cells (28). Blockade of DHODH activity could inhibit the pyrimidine synthesis and the tumorigenic capacity of GBM stem cells (29). However, whether the suppression on proliferation of GBM cells caused by DHODH inhibition is associated with ferroptosis still needed to be explored. The role and regulatory mechanism of DHODH downregulation in LIHC are also needed to be studied.

In this current study, DHODH overexpression was found to be linked with improved OS in KIRP and decreased OS in SARC. A previous study reported that LEF which was an inhibitor of DHODH significantly impeded the proliferation of renal carcinoma cells and tumor growth in an animal model through suppressing the canonical Wnt/β-catenin signaling pathway (30). Whether the dysregulation of DHODH is linked with ferroptosis of renal carcinoma cells and the role of DHODH in SARC need to be studied in the future.

The current data indicated that the mRNA expression level of DHODH was higher in *TP53*-mutated BLCA, ESCA, HPV-negative HNSC, and luminal-A BRCA patients and in *PTEN*-mutated KIRP, PRAD, UCEC, and BLCA patients. These findings suggest that there might be regulatory connections between DHODH expression and mutations of *TP53* and *PTEN*. Tenovins was reported to activate p53 and kill cancer cells by inhibiting DHODH activity and blocking uridine transport into cells (31). The inhibitors of DHODH, tetrahydroindazoles, could suppress cancer cell growth and viability by activating p53 and its downstream genes (32). DHODH inhibitors could enhance the p53 synthesis and promote cancer cell death through blocking p53 degradation (33). Inhibition of DHODH could also reduce ATP level and increase the reactive oxygen species (ROS) level of breast cancer cells via upregulating p53, p65, and STAT6, and finally inhibit the proliferation of cancer cells (34). All these findings highlight DHODH as an upstream regulatory gene in the p53 signaling pathway, however, the expression level of DHODH in p53 mutant cancer cells is still unclear and needs to be evaluated. A previous study reported that mutations in *PTEN* generated different dihydroorotase (a rate-limiting step enzyme in pyrimidine synthesis) phosphorylation patterns and further activated carbon influx by regulating pyrimidine synthesis in GBM stem cells (29). Nevertheless, the regulatory mechanism underlying mutation of *PTEN* and DHODH expression or activity is still unknown.

Recently, a study reported that combination of the DHODH inhibitor LEF with RGX-202 (an inhibitor of SLC6A8) suppressed CRC growth in animal models (35). Importantly, our results further showed that knockdown and DHODH inhibitor LEF suppressed the cell proliferation, colony formation, and cellular ATP levels in ESCC and CRC cells. Interestingly, the apoptosis inhibitor partially rescued LEF-induced cell death of ESCC cells; moreover, ferroptosis and necroptosis inhibitors partially rescued LEF-induced cell death of CRC cells, respectively. These results suggest that LEF induced apoptosis of ESCC cells and led to ferroptosis and necroptosis of CRC cells. Up to now, the roles and mechanisms of DHODH in cell death, especially the necroptosis and ferroptosis of tumor cells, are almost completely unknown, thus, future studies should

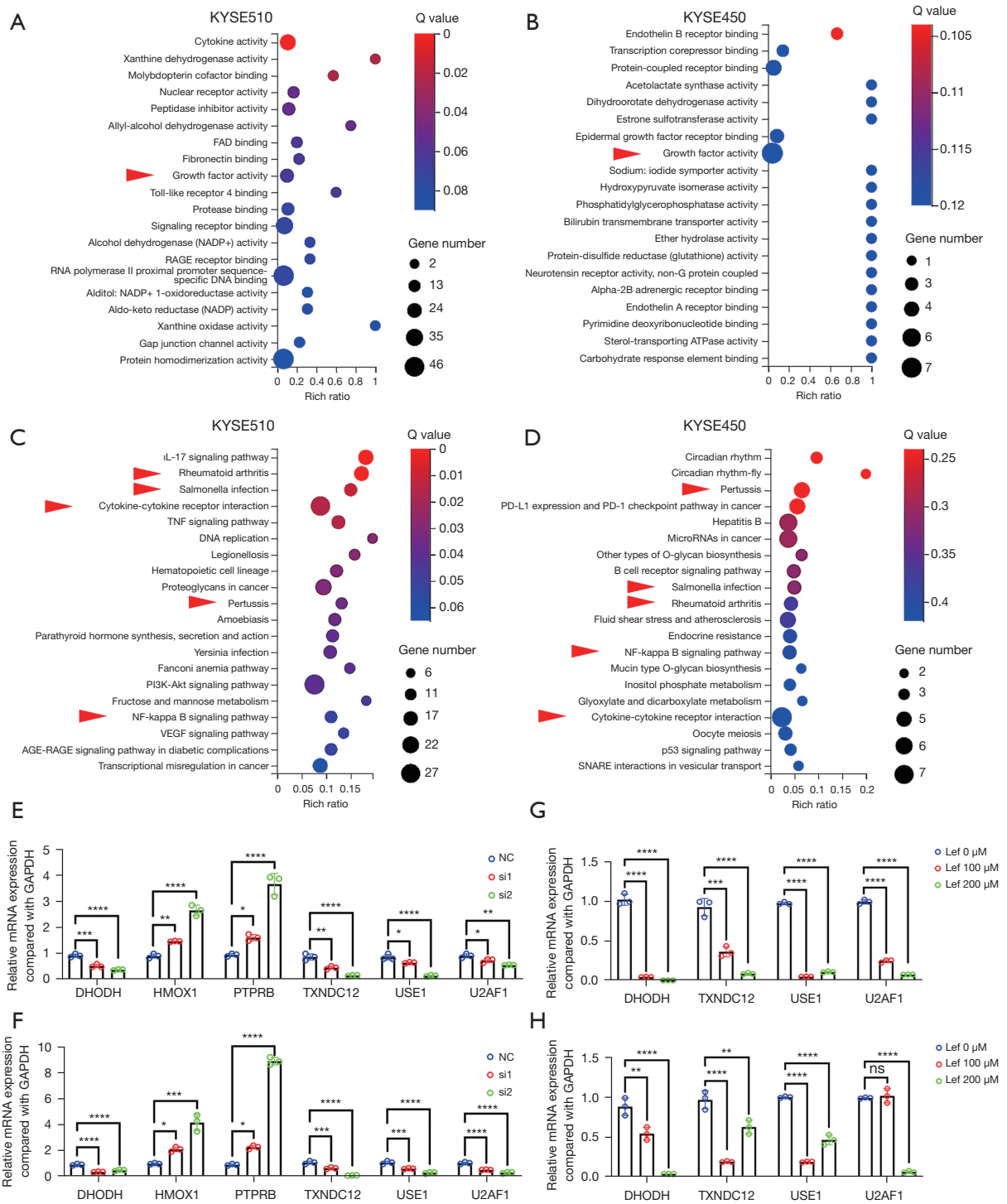

Figure 6 Identification of downstream pathways of DHODH in ESCC. (A,B) MF analysis based on DEGs after DHODH knockdown in KYSE510 and KYSE450 cells. (C,D) KEGG analysis based on DEGs after DHODH knockdown in KYSE510 and KYSE450 cells. (E-H) qRT-PCR method was used to validate the DEGs identified by RNA-seq on the conditions of DHODH knockdown and LEF treatment. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. Arrowhead, enrichment in both KYSE510 and KYSE450 cells. DHODH, dihydroorotate dehydrogenase; ESCC, esophageal squamous cell carcinoma; MF, molecular function; DEG, differentially expressed gene; KEGG, Kyoto Encyclopedia of Genes and Genomes; qRT-PCR, quantitative reverse transcription polymerase chain reaction; LEF, leflunomide.

aim to explore the regulatory roles of DHODH during ferroptosis and necroptosis of cancer cells.

Conclusions

Taken together, our results indicate that DHODH is dysregulated in many kinds of tumors including ESCA, COAD, and READ. Importantly, DHODH was shown to suppress apoptosis in ESCC and inhibit ferroptosis and necroptosis in CRC. Whether a therapeutic method of targeting DHODH-induced ferroptosis could be used to treat DHODH-overexpressed cancers needs to be explored in future.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-136/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-136/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The Medical Ethics Committee of Kunming University of Science and Technology approved this study (No. KMUST-MEC-123) and informed consent was provided by all the patients.

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